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**This is the author's manuscript**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/86300> since 2016-11-23T12:27:58Z

*Published version:*

DOI:10.1038/leu.2011.12

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**IGHV unmutated CLL cells are more prone to spontaneous apoptosis and subject to environmental prosurvival signals than mutated CLL cells**

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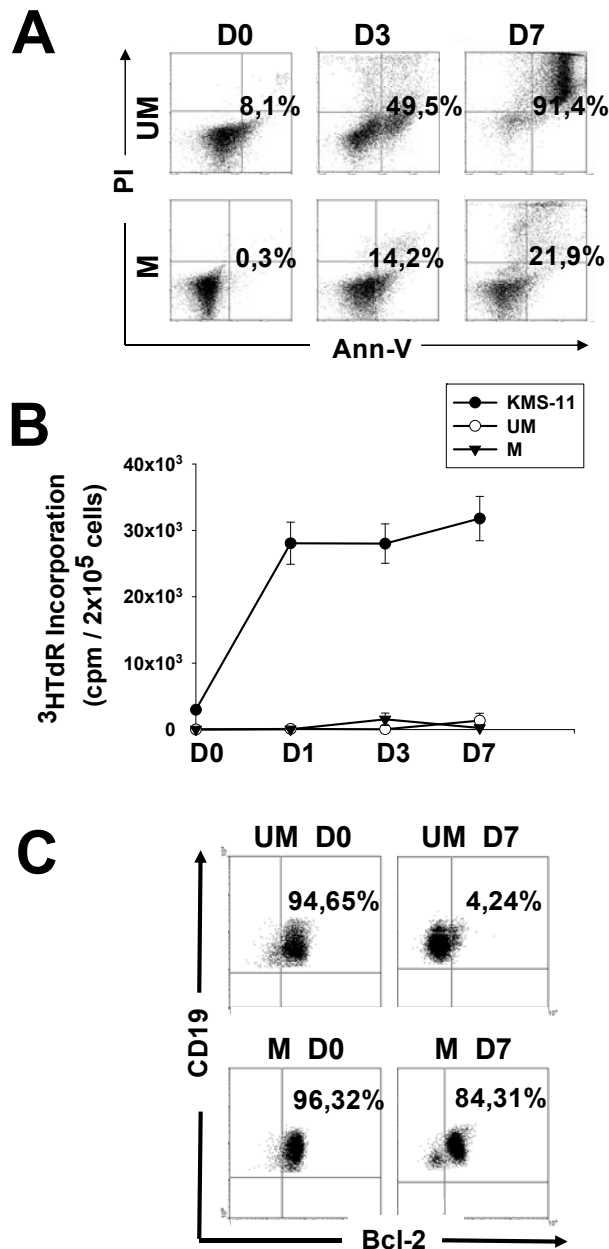
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## **Abstract**

Tumor cells in Chronic Lymphocytic Leukemia (CLL cells) are more prone to apoptosis when cultured *ex vivo* because they lack prosurvival signals furnished *in vivo* via B-cell receptor (BCR)-dependent and -independent pathways. This study compared the susceptibility of unmutated (UM) and mutated (M) CLL cells to spontaneous apoptosis and prosurvival signals. UM CLL cells showed a significantly higher rate of spontaneous apoptosis than M CLL cells. Nuclear factor- $\kappa$ B (NF- $\kappa$ B) was rapidly inactivated and Bcl-2 expression progressively down-regulated in the UM CLL cells. CD40-Ligand, interleukin-4 and stromal cells significantly improved their viability and partially recovered B-cell leukemia/lymphoma 2 (Bcl-2), but not NF- $\kappa$ B expression. Peripheral blood mononuclear cells also offered protection of UM CLL cells and recovered both NF- $\kappa$ B and Bcl-2 expression. T cells, rather than nurse-like cells, were responsible for protecting UM CLL cells by means of cell-to-cell contact and soluble factors. The more aggressive features of UM CLL, which stem from their enhanced BCR transducing capacity, are subject to prosurvival signals, which instead have minimal effect on M CLL cells, whose slower apoptotic rate remained unaltered. This vulnerability of UM CLL cells can be exploited as a selective target of therapeutic interventions.

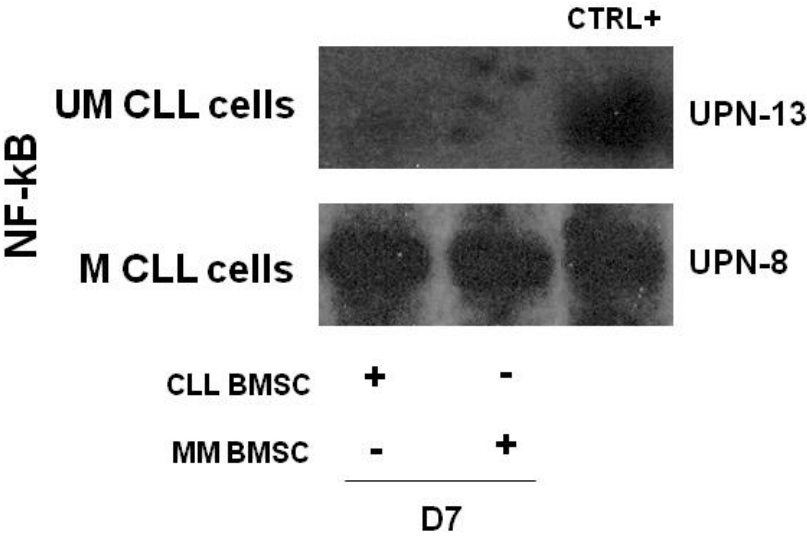
M.C. and F. P. contributed equally to this study.

## SUPPLEMENTAL FIGURE 1



**Apoptosis, proliferation and Bcl-2 expression in M and UM CLL cells.** (A) Representative analysis of Ann-V/PI staining after backgating on CD19<sup>+</sup>/CD5<sup>+</sup> UM and M CLL cells at the indicated time intervals. (B) In vitro proliferation was assessed by measuring [<sup>3</sup>H]TdR incorporation at day (D)0, D1, D3 and D7, and expressed as cpm/2x10<sup>5</sup> cells. Lines represent the mean  $\pm$  SEM [<sup>3</sup>H]TdR incorporation of CLL cells purified from UM (n=3) and M (n=3) patients. M CLL cells did not display higher [<sup>3</sup>H]TdR incorporation than UM CLL cells. The KMS-11 cell line was used as a positive control. (C) Representative analyses of intracellular Bcl-2 expression after backgating on CD19<sup>+</sup>/CD5<sup>+</sup> CLL cells, at D0 and D7.

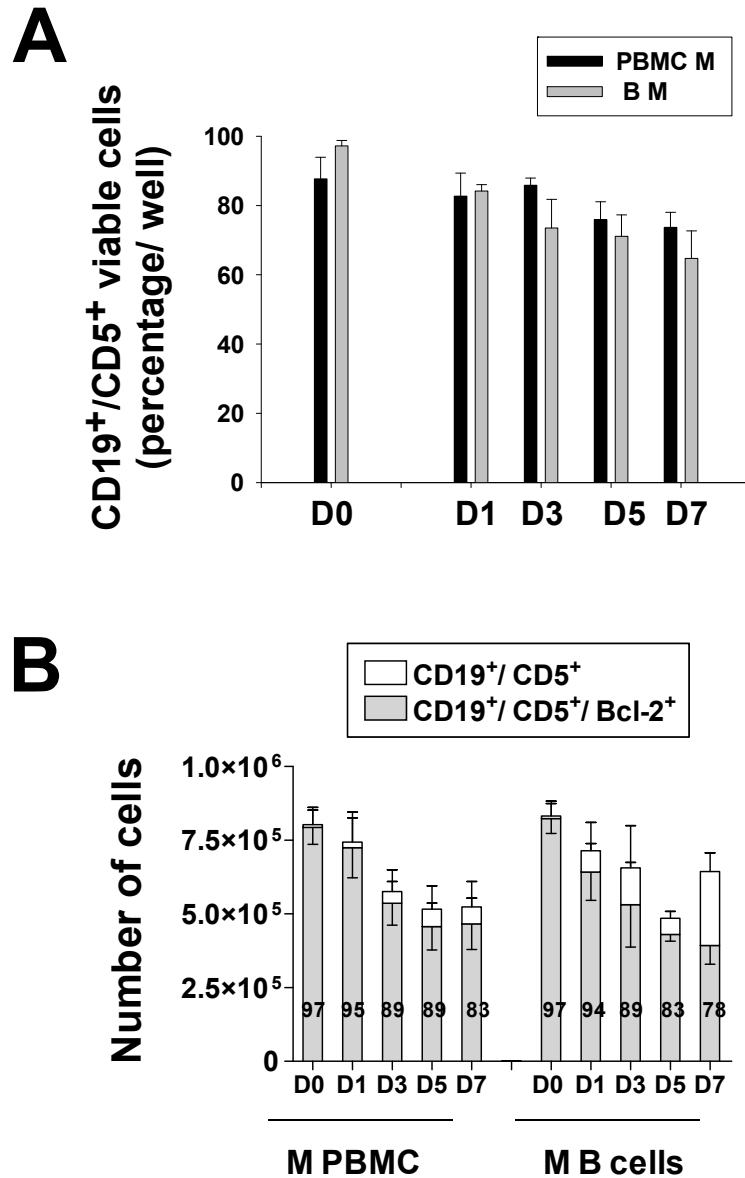
**SUPPLEMENTAL FIGURE 2**



**NF-κB expression in purified M and UM CLL cells co-cultured with stromal cells.**

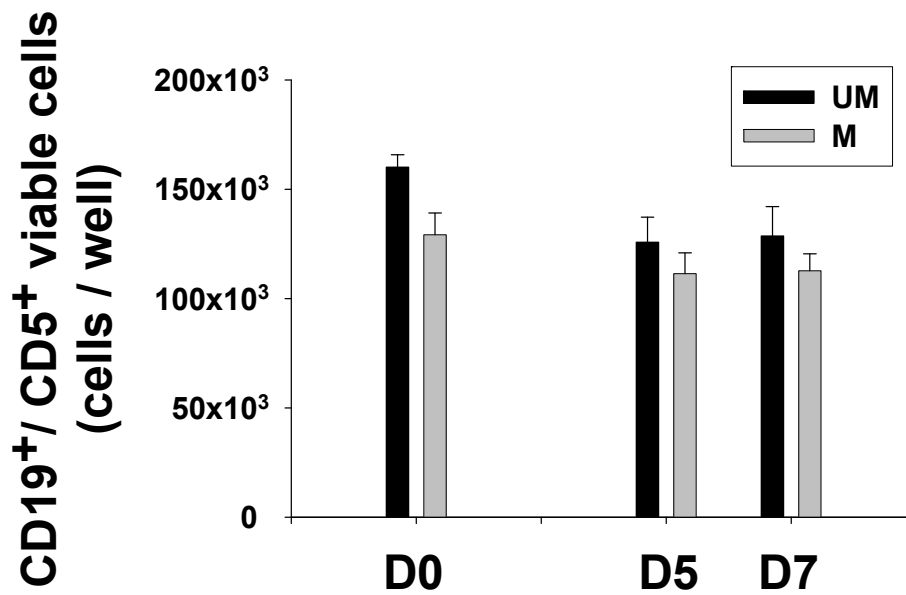
Bone marrow stromal cells (BMSC) derived from CLL and multiple myeloma (MM) patients did not affect NF-κB nuclear translocation in M and UM CLL cells after 7 days of culture. Results are from 1 UM and 1 M representative patients (UPN, unique patient number).

**SUPPLEMENTAL FIGURE 3**



**In vitro survival and Bcl-2 expression in purified M CLL cells and M PBMC.** (A) Percentage of CD19<sup>+</sup>/CD5<sup>+</sup> viable CLL cells. Bars represent the mean  $\pm$  SEM of 11 (M CLL cells) and 17 (M PBMC) experiments. The percentage of Ann-V and PI negative cells within CD19<sup>+</sup>/CD5<sup>+</sup> CLL cells was evaluated at D0 and at D1, D3, D5 and D7. The difference in cell viability of M CLL cells and M PBMC was not statistically significant. (B) Bcl-2 expression in isolated M CLL cells versus M PBMC. White bars represent the absolute number of CD19<sup>+</sup>/CD5<sup>+</sup> cells/well, gray bars represent numbers of CD19<sup>+</sup>/CD5<sup>+</sup>/Bcl-2<sup>+</sup> cells/well. Within bars, the percentage of Bcl-2<sup>+</sup> CLL cells is indicated. Differences between M CLL cells and M PBMC were not statistically significantly.

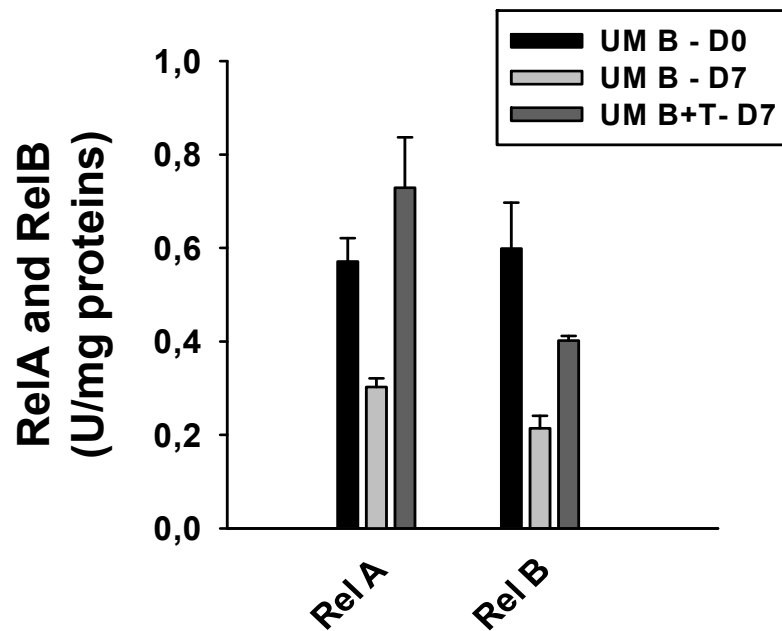
#### SUPPLEMENTAL FIGURE 4



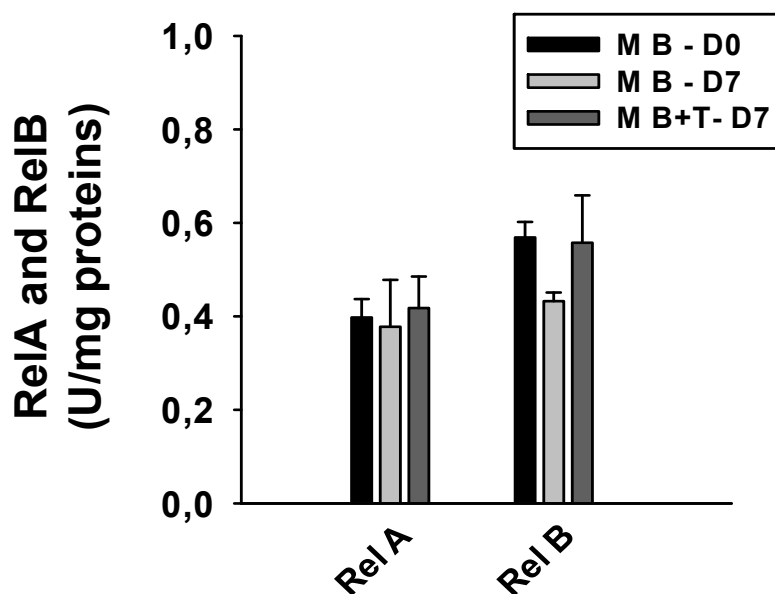
**Viability of M and UM CLL cells within the non-adherent fraction of PBMC.** Cell viability was detected by evaluating Ann-V/PI expression after backgating on CD19<sup>+</sup>/CD5<sup>+</sup> cells. Staining was performed on the non-adherent fraction of PBMC from M (grey bars) and UM (black bars) patients at D0, D5 and D7 of culture. Differences in the mean number of viable CD19<sup>+</sup>/CD5<sup>+</sup> cells in M and UM samples were not statistically significant.

**SUPPLEMENTAL FIGURE 5**

**A**



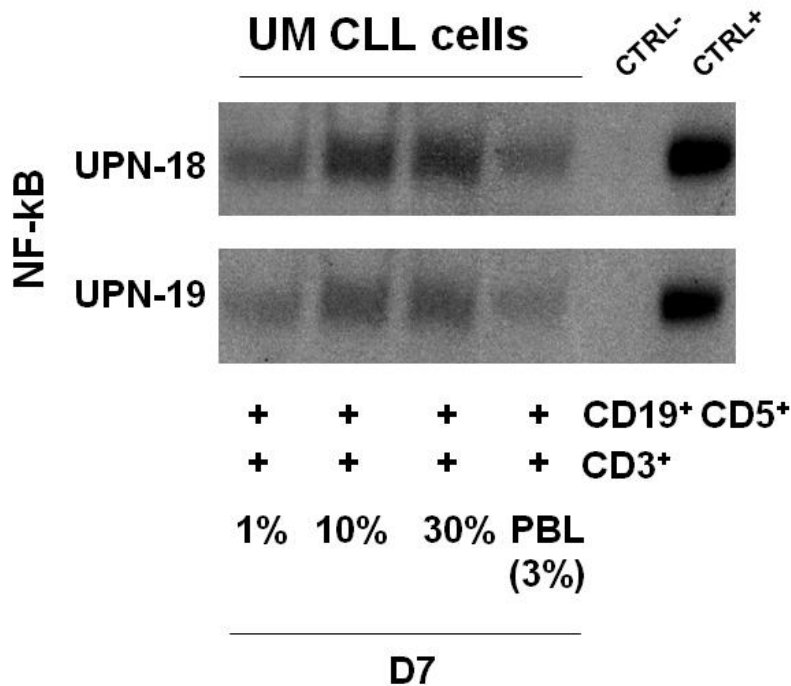
**B**



**RelA and RelB NF-kB subunits in B cell/T cell co-cultures.** Quantitative analysis of RelA and RelB NF-kB subunits was performed using a DNA binding ELISA-based method. On D7, both RelA and RelB levels were reduced in nuclear extracts from purified UM CLL cells, whereas they were up-regulated by the presence of autologous T cells. As shown, in M CLL cells RelA and RelB levels did not change during the 7-day culture, and they were impervious to the presence of T cells.

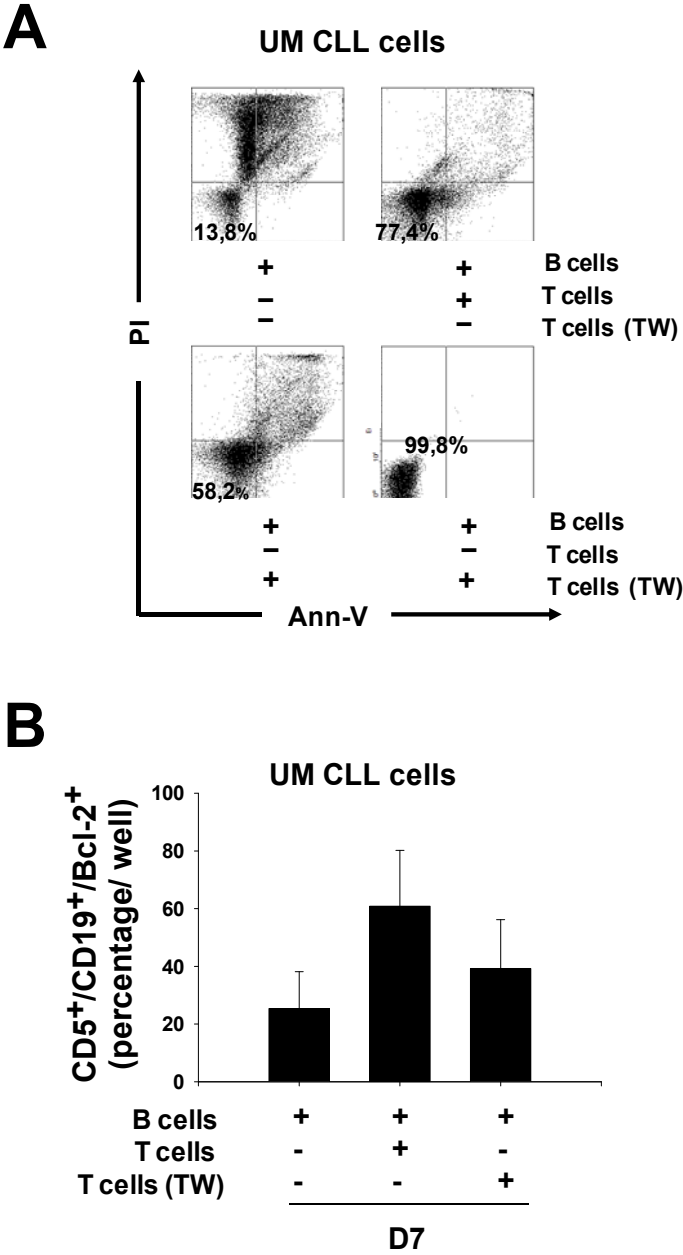


**SUPPLEMENTAL FIGURE 6**



**NF-kB expression in nuclear extracts of UM CLL cells exposed to increasing quantities of autologous T cells.** Titration experiments were performed by co-culturing UM CLL cells with increasing quantities of autologous T cells. As shown, there was a dose-dependent modulation of NF-kB activity, detected by EMSA. NF-kB expression in peripheral blood lymphocytes (PBL), which were obtained by removing the monocyte adherent fraction of PBMC, was also evaluated.

# SUPPLEMENTAL FIGURE 7



**Apoptosis and Bcl-2 expression in UM CLL cells cultured alone, in contact with T cells and separated from T cells by the presence of micropore inserts (TW). (A)**

Representative analyses of Ann-V/PI expression after backgating on CD19<sup>+</sup>/CD5<sup>+</sup> UM CLL cells on D7. A representative dot plot of Ann-V/PI staining on purified T cells is also shown (lower-right quadrant). The percentages of Ann-V/PI double negative viable CLL cells are indicated.

(B) Bcl-2 expression in UM CD19<sup>+</sup>/CD5<sup>+</sup> CLL cells at D7. Bars

represent the mean  $\pm$  SEM of 8 experiments. As shown, Bcl-2 expression was partially

restored by the presence of autologous T cells. The protective effect exerted by cell-to-cell contact was stronger compared to TW conditions. None of these differences reached statistical significance.

# SUPPLEMENTAL TABLE 1

**TABLE 1. CK concentration ranges**

<i>CK</i>	<i>UM. pg/ml</i>	<i>M. pg/ml</i>
IL-1RA	0.87 - 20.54	0.87 - 154.02
IL-6	0.06 - 0.97	0.06 - 13.13
IL-8	0.59 - 236.40	3.33 - 381.62
IL-15	1.65 - 12.90	1.65 - 21.16
IL-17	1.94 - 3.97	2.1 - 11.61
IP-10	4.03 - 58.76	4.03 - 110.35
MCP-1	5.48 - 1 534.63	5.02 - 8 412.50
MIP-1a	1.37 - 5.67	1.60 - 130.80
MIP-1b	28.1 - 290.10	29.03 - 1 271.86
PDGF-BB	0.47 - 2.58	0.59 - 2.65
RANTES	2.44 - 4.67	7.86 - 23.80
TNF-a	0.22 - 3.10	0.16 - 6.09
VEGF	0.23 - 4.01	1.35 - 1.9

Concentration range of interleukin (IL)-2, IL-4, IL-7, IL-10, IL-12, IL-13: 0 - 0.5

Concentration range of fibroblast growth factor (FGF), Interferon-gamma (IFN-g), IL-1 $\beta$ , IL-5, IL-9, granulocyte-macrophage colony-stimulating factor (GM-CSF), Granulocyte colony-stimulating factor (G-CSF), Eotaxin: 0.5 – 2

CK indicates cytokines; IL, interleukin; IP-10, interferon-inducible protein-10; MCP-1, Monocyte chemotactic protein-1; MIP-1a, Macrophage inflammatory protein 1 alpha; MIP-1b, Macrophage inflammatory protein 1 beta; PDGF, platelet-derived growth factor; RANTES, Regulated on Activation Normal T Cell Expressed and Secreted; TNF-a, tumor necrosis factor-alpha; VEGF, Vascular endothelial growth factor.